DRY HEAT DESTRUCTION RATES OF MICROORGANISMS ON SURFACES AS A FUNCTION OF RELATIVE HUMIDITY

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INTRODUCTION

The plan to use a dry nitrogen environment for terminal sterilization in the Viking '75 mission has created an immediate need for information concerning surface spore-destruction rates under dry-nitrogen conditions. Considerable data are available concerning dry heat destruction rates of spores (particularly Bacillus subtilis var. niger) which have been equilibrated and heated in ambient clean-room environments. Since the Viking '75 lander will be sterilized in a dry-nitrogen atmosphere, it is imperative to obtain comparable data for that environment. In this regard, a test system has been advised and a series of experiments has been initiated to obtain D-values for spores in a dry gas (either air or nitrogen) as well as in clean room environments.

OBJECTIVE

The specific objective of this project was to determine dry heat destruction rates (D-values) for several species of microbial spores deposited on surfaces and subjected to temperatures in the range of 90°-125°C as a function of relative humidity and types of atmosphere in contact with the spores.

EXPERIMENTAL PROCEDURE

Microbial Spores

In the experiments carried out thus far, Bacillus subtilis var. niger spores obtained from suspensions with several different

histories have been utilized. These were as follows:

Suspension Spore Code AAAB: Spores obtained from the Phoenix NCDC Laboratory approximately one year earlier, regrown in our laboratory (growth procedure #1), stored in ethanol and then re-suspended in deionized distilled water.

Suspension Spore Code AANE: Spores obtained from the Phoenix NCDC Laboratory (procedure #2) which had been stored in buffered distilled water and were then re-suspended in deionized distilled water.(A new crop of this spore was received later in a frozen condition and was designated as AAOE.

Suspension Spore Code AADB: Spores recently harvested from the Phoenix Laboratory crop,(procedure #1) stored in ethanol, and then re-suspended in deionized distilled water.

(See Appendix B for details of spore preparation.)

Heating System

Spore deposits were made on 1/2" x 1/2" stainless steel planchets previously placed on rectangular copper boats allowing five strips/boat (See Appendices C and D). In all experiments carried out thus far, spores have been heated at 110°C on a specially designed hot plate. The temperature was determined using thermocouples attached to the copper boats; two boats with thermocouples were placed on each of the hot plates. The thermocouples were connected to a temperature recording potentiometer so that we had a record of the temperature of the hot plate during the experiment. For each condition tested, five boats were heated; one additional boat was always processed after equilibration (but before heating) for determination of N_o and a second additional boat was carried along as a spare. One randomly allocated boat for each condition was removed at each of five time-intervals for processing. In all instances, four planchets from each boat were processed according to the standard procedure. The fifth planchet (the middle one) was not processed unless one of the other four was inadvertently mishandled. This procedure was initiated to minimize the possibility of missing data. Additional control procedures related to plating media consistency are outlined in Appendix F.

Gas Control System

In all experiments, heating was carried out in the special dry gas environment and in a laminar crossflow clean room used as a control. The clean room ran consistently at 22-24°C and 34-44% RH during the experimental period. During heating the spore deposits were protected from the direct air stream by a perforated plastic box. (See Figure 5.1)

The dry gas environment was set up in a double glove-box arrangement (Figures 5.2 and 5.3). One box was used for equilibration and the other for heating; each had its own access antechamber. In experiments thus far we have utilized either predried dry air or dry nitrogen from compressed gas tanks to pressurize the two glove-box chambers independently. A bellows arrangement maintained a consistent positive pressure in the two chambers and their access chambers. A Bell and Howell (C.E.C.) moisture analyzer was connected to the apparatus so that a direct readout of moisture content could be obtained from either chamber. During experiments thus far, relative humidity has ranged from 0.1 to 2.0% at 25°C in the chamber air. Figure 5.4 outlines the basic glove-box set-up.

The initial experiments in this project were designed to determine the basic workability of the apparatus and to select suitable specific-equilibration times, spore suspensions, and initial concentrations of spores. In addition they were used to finalize specific processing techniques and equipment operation procedures, to aid in the selection of suitable time intervals and plating dilutions and to perfect computer-analysis programs. While much valuable information has resulted from the initial experiments, the specific procedures have necessarily been somewhat variable.

All experiments carried out thus far at 110°C can be summarized as follows:

1. Comparison of the dry heat destruction rates of the three spore suspensions (AAAB,AANE, AADB) as a function of relative humidity of the gas surrounding the spore deposit.
2. Comparison of the dry heat destruction rates as a function of equilibration time and relative humidity.

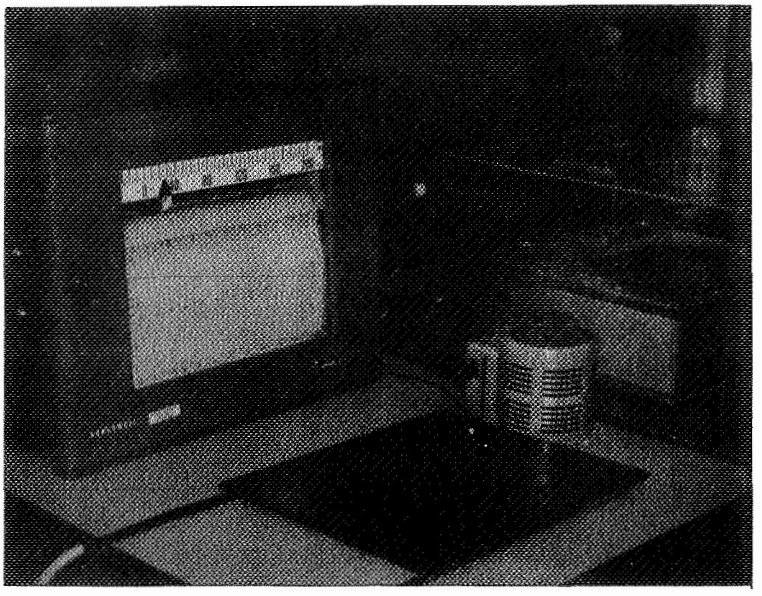

Figure 5.1 - Hot plate, cold plate and temperature recorder in the clean room

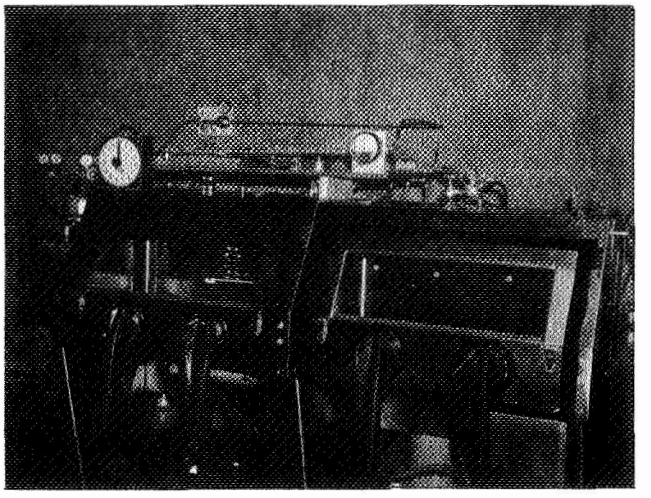

Figure 5.2 - Two-chamber glove box and auxiliary equipment

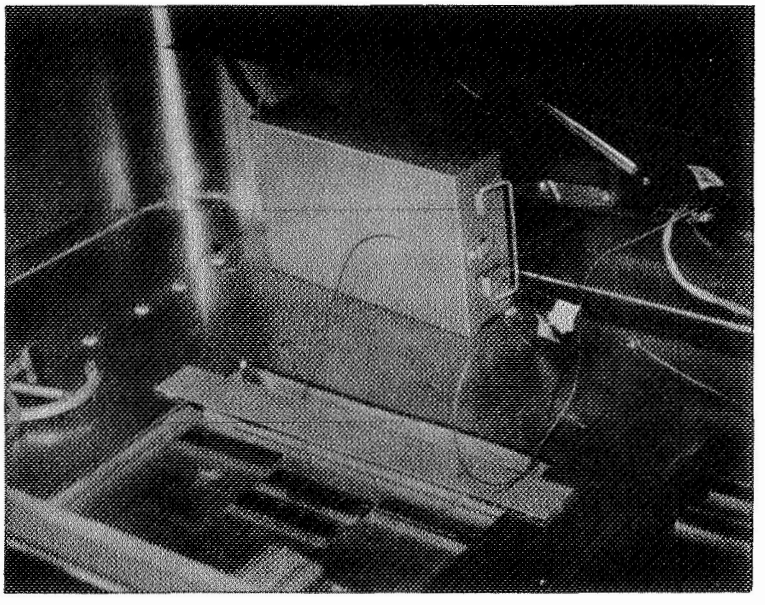

Figure 5.3 - Hot plate built into the bottom of the glove box with boats in place on the hot plate

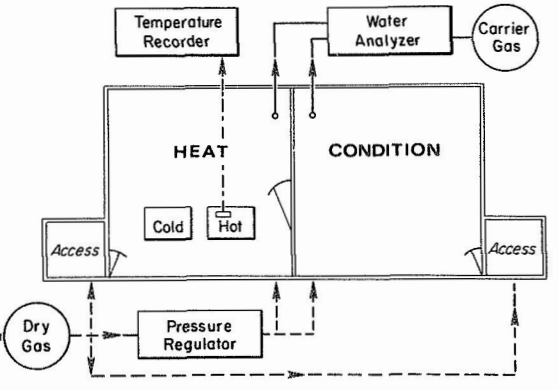

Figure 5.4 - Schematic diagram of the glove box system

3. Comparison of the dry heat destruction rates of a frozen and a non-frozen spore suspension.
4. Comparison of dry heat destruction rates as a function of initial spore concentration per volume of water per deposit.
5. On the basis of information from the above experiments a series of experiments at 110°C was initiated to complete the objectives of the project for that temperature and was designed as follows:*

Experiment A: Using dry air for the <1% heating and equilibration

a. Spores were equilibrated at <1% RH and heated at <1% RH
b. Spores were equilibrated at ∿35% RH and heated at <1% RH
c. Spores were equilibrated at <1% RH and heated at ∿35% RH
d. Spores were equilibrated at ∿35% RH and heated at ∿35% RH

Experiment B: Using dry nitrogen for the <1% heating and equilibration

a. Spores were equilibrated at <1% RH and heated at <1% RH
b. Spores were equilibrated at ∿35% RH and heated at <1% RH
c. Spores were equilibrated at <1% RH and heated at ∿35% RH
d. Spores were equilibrated at ∿35% RH and heated at ∿35% RH

*In both experiments the RH was measured at 22-25°C.

Spores AAOE with an equilibration time of 24 hours, were used in all tests. Each experiment was performed at least two times. Details of the experimental procedure follow.

General

All operations (prior to incubation of the plates) except those performed in the dry gas chamber, were carried out in a clean room. The dry gas chamber was located immediately adjacent to the clean room. Aseptic technique was used at all times. Freshly laundered clean-room coats and hats were worn in the clean room when performing microbiological tasks or upon entering the clean room when experimental work was being carried out.

Twenty (5 ml) aliquots of the spore suspension coded AAOE (See Appendix B) were placed into sterile glass vials, covered tightly and stored at 4°C for use in these experiments. A different vial was

used for each trial. After spore deposits were made, the vial was covered, dated, and stored at 4°C. If problems were encountered, it would be possible to go back to the exact spore suspension for further investigation.

Specific Method

1. Copper boats were arranged in order on a stainless steel sheet on the table in the clean room and spore deposits were made.*
2. When all spore deposits were made, a metal air deflector, which was closed on the top and three sides, was placed over the stainless steel sheet on which the copper boats were arranged. The closed end of the deflector faced the HEPA filter wall. The deposits were allowed to dry until no visible water remained. Conditioning time in the clean room (35% RH and 25°C) began when the spore deposits appeared visibly dry.
3. The copper boats to be conditioned at <1% RH, were put into two separate metal transfer boxes, one for each of two treatment groups (<1% RH conditioning and heating and <1% RH conditioning and 35% heating). The boats to be conditioned at 35% RH remained on the stainless steel sheet, under the air deflector.
4. The transfer device (See Figure 5.5, page 58), which was used to transfer the copper boats conditioned at <1% RH and those to be heated at <1% RH, was placed on its side in the conditioning chamber (right side) of the double-chambered, environmentally controlled glove box outside of the clean room. The hood was flushed until the RH was less than 1% (overnight). The two metal transfer boxes to be conditioned at <1% RH were placed in the pass-thru of the conditioning chamber. The transfer box covers were then opened and the pass-thru door was closed. The pass-thru was then flushed for thirty minutes with the same type of gas being used in the test system, (either dry air

* See Method of Randomization of Treatment Combinations and Spore Deposit Method (Appendices C and D)

or dry nitrogen); this involved about ten gas exchanges. Immediately before and after the transfer boxes were placed in the conditioning chamber, the water content of the gaseous environment of the conditioning chamber was determined. Results were then compared to check the efficiency of the flushing method. Conditioning time (24 hours) began when the metal boxes were transferred from the pass-thru into the conditioning chamber.

5. After 24 hours conditioning, the pass-thru of the conditioning chamber was flushed again. By means of the special transfer device (Figure 5.5) the boats conditioned at <1% RH and those to be heated at 35% RH were transferred thru the pass-thru to the hot plate in the clean room. All boats were simultaneously transferred from the stainless steel sheet to the hot plate. The boats conditioned at 35% RH and those to be heated at 35% RH were placed on the hot plate by means of the holding tool (See Figure 5.6) The water content of the gas in the chamber was determined before and after opening the pass-thru door to effect the transfer.
6. Using the special transfer device, the copper boats conditioned at 35% RH and those to be heated at <1% RH were transferred into the pass-thru of the conditioning chamber. The pass-thru was flushed and the transfer device was placed in the conditioning chamber. Again the water content of the gas in the chamber was determined before and after opening the pass-thru.
7. The heating chamber (left side) of the double-chambered, environmentally controlled hood was flushed until the RH was less than 1%. The temperature of the chamber was approximately 25°C, and was maintained by cooling coils located inside of the chamber. A thermocouple located six inches above the surface of the hot plate gave a continuous read-out of the chamber temperature.

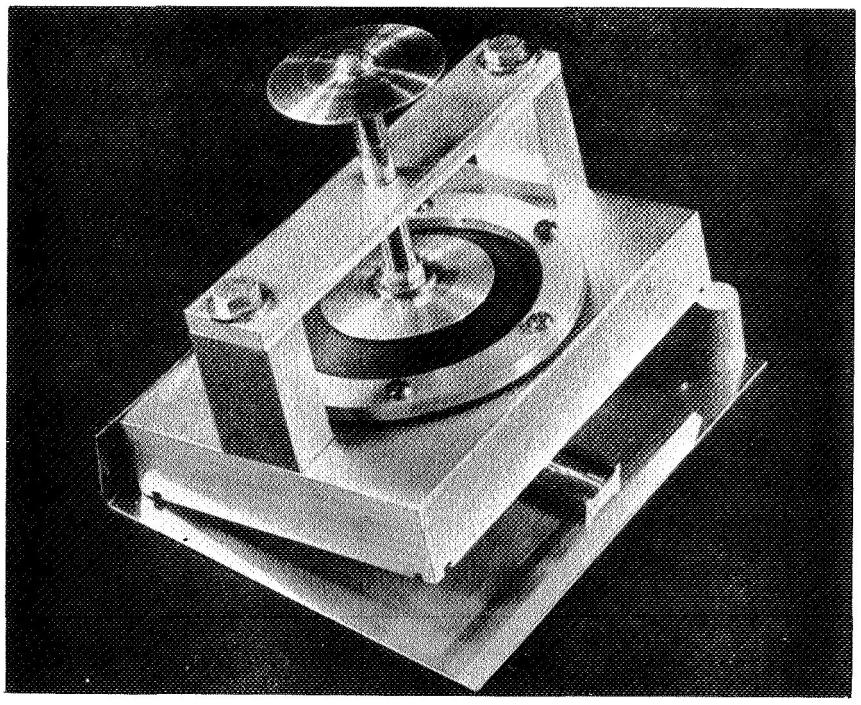

Figure 5.5 - Transfer device

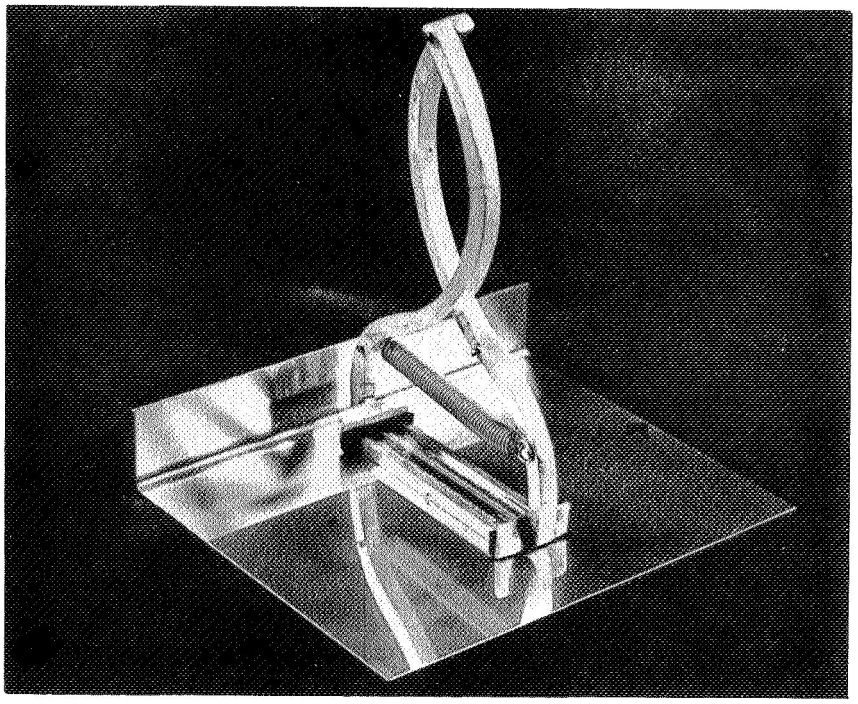

Figure 5.6 - Boat-holding tool

8. The metal transfer box (with strips conditioned at <1% RH and the transfer device (with strips conditioned at 35% RH) were passed from the conditioning chamber into the heating chamber via the inter-chamber pass-thru. The water content of the gas was determined before and after opening the pass-thru door.
9. The copper boats were moved to the hot plate simultaneously from the stainless steel sheet of the transfer device. The boats from the transfer box were placed on the hot plate using the special holding tool (Figure 5.6).
10. Two copper boats which were not involved in the experiment were located on the hot plates in the clean room and heating chamber, one on the upper-left and one on the upper-right corner of each hot plate. They were attached by means of thermocouples to a continuous multichannel recording potentiometer. The pass-thru doors of the heating chamber were never opened until all of the specified heating times had been completed. Heating intervals in the dry gas environment were 25, 50, 75, 100, and 125 minutes. In the clean room they were 85, 170, 255, 340, and 425 minutes for the 110° experiments.
11. Immediately upon completion of each heating interval the designated boat was placed on the cooling plate for a minimum of three minutes. The special holding tool was used to transfer boats. A stainless steel sensing disk was laid on the surface of the cooling plate which was connected by means of a thermocouple to the continuous multi-channel recording potentiometer.
12. All five heating times were completed before processing the strips. The boats were placed in their respective boxes and transferred to the clean room for processing.
13. The copper boat was picked up by means of the special holder and transferred to a sterile-gloved hand. Each of four replicates (planchets) was transferred with a sterile forceps (same one for four replicates from a single boat), spore-side down into a dry, sterile, pre-labeled 125 ml Erlenmeyer Flask. The fifth (center) planchet was not analyzed but was retained in its respective boat in case another strip was lost. If time

did not permit immediate processing the strips were stored dry in their respective labeled flasks until processed. Strips were processed the morning following the heating treatment, but no later than that.

14. To begin processing, 50 ml of sterile phosphate buffer was added to each flask. (Actual observation revealed that the bottles contained 50 ±1 ml of buffer.
15. A single flask, containing the strip and buffer solution was suspended in the ultrasonic tank using a rubber-coated, Stoddard-type test tube holder attached to the wall of the bath as the support. The bottom of the flask was parallel to the bottom of the tank and the tank solution (0.3% V/V Tween 80, aqueous) was level with the buffer solution in the flask.
16. Each flask was insonated at 25 Khz per second for two minutes.
17. Three different aliquots were plated in duplicate, using 15 x 100 mm disposable petri plates and disposable pipettes. Very small volumes (0.01 and 0.05 ml) were measured with a 10 µl or 50 µl Eppendorf Microliter Pipette. Five and ten aliquots were plated using 15 ml of one-and-one-half strength Trypticase Soy Agar (TSA); all other volumes were plated with 20 ml single-strength TSA. The expected D-value was used as the basis for selecting the heating time and the volumes to be plated so the results would yield countable plates at each time interval, with the last heating time calculated to yield approximately 1×10^2 spores per strip.
18. Plates were inverted and incubated for 48 hours at 32°C and counted with the aid of a Bactronic Counter. A single duplicate plate from each aliquot of each treatment combination was counted by each of two counters. If more than two counters were used, then the plates to be counted by each counter had to be randomly allocated.
19. Preliminary D-values and y-intercepts were determined by plotting (on semilogoarithmic paper), the number of survivors (on the logarithmic scale) versus the heating time in minutes (on the linear scale) and drawing a "best fit" line using the number of survivors at the five heating times but not N_o.

20. The estimated number of surviving spores at each heating time was recorded and forwarded to the biomedical computer center for computer analysis. The computer program utilized a least squares regression analysis (again ignoring N_o) to estimate a D-value and y-intercept for each time-temperature treatment combination.

RESULTS

Experiment 1

Results of the comparison of the $D_{110°C}$-values of the three spore suspensions are summarized in Table 5.1, while typical survivor curves for each suspension are shown in Figures 5.7, 5.8, and 5.9. It is obvious that regardless of which suspension was used, the dry gas environment resulted in significantly lower (about four-fold) $D_{110°C}$-values than did the ambient clean room environment. Of the three suspensions the water-stored spores and the new preparation of ethanol spores were more resistant than the older ethanol-stored spores.

Table 5.1 - $D_{110°C}$-values of Three *Bacillus subtilis* var. *Niger* Suspensions as a Function of Relative Humidity

Heating Environment	% RH Range		$D_{110°C}$-Value in Min. (No. of Trials)		
	At 25°C	At 110°C	Spores AAAB	Spores AANE	Spores AADB
Dry Gas Box (using dry air)	0.10-1.70	0.018-0.025	21.1 (2)	32.5 (4)	32.7 (8)
Laminar Crossflow Clean Room	40-42	0.89 0.93	99.3 (4)	145.4 (8)	121.3 (2)

Experiment 2

The effects of equilibrium time on the $D_{110°C}$-values of the three spore suspensions in both the dry-gas and clean-room environments are shown in Table 5.2. Survivor curves for the several conditions are shown in Figure 5.10. In all cases the spores were equilibrated in the same environment in which they were heated. Extension of the equilibration time did

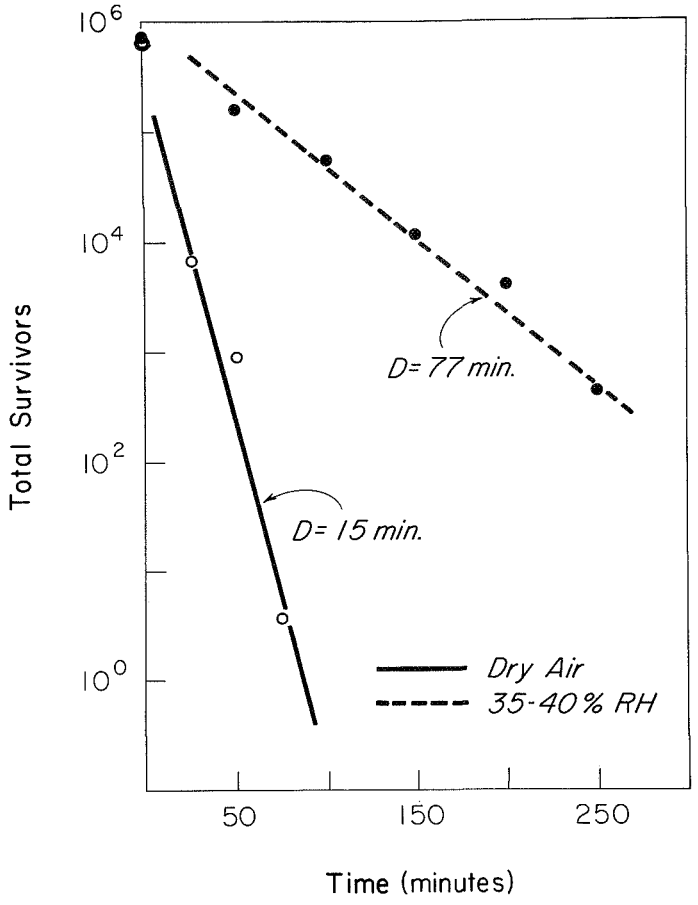

Figure 5.7 - Survivor curves for spores AAAB in dry and 35-40% RH air.

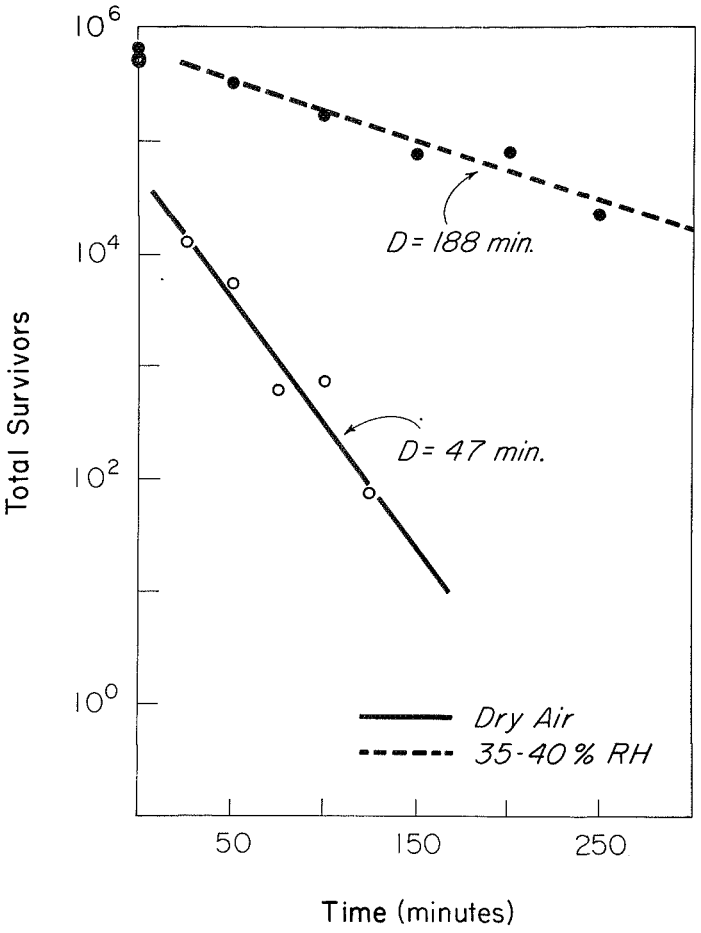

Figure 5.8 - Survivor curves for spores AANE in dry and 35-40% RH air.

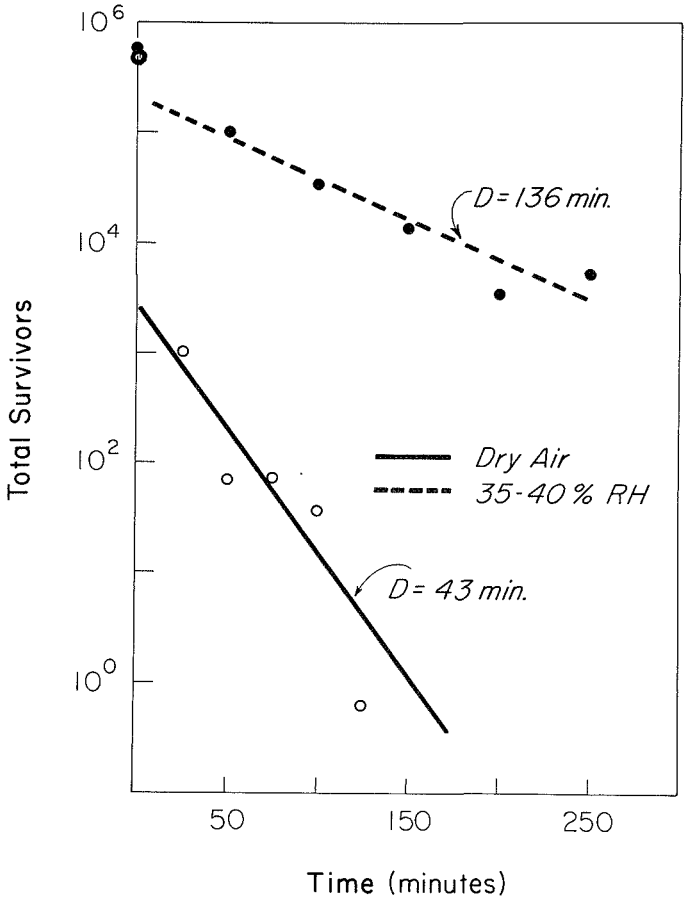

Figure 5.9 - Survivor curves for spores AABD in dry and 35-40% RH air.

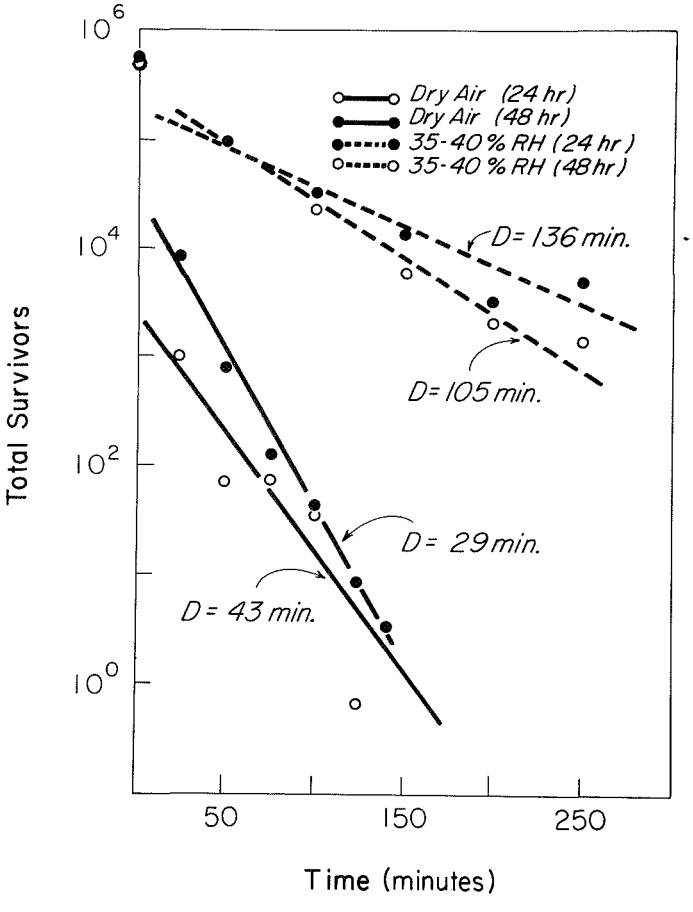

Figure 5.10 - A comparison of 24 and 48 hour equilibration for spores AABD in dry and in 35-40% RH air.

not affect the $D_{110°C}$-value significantly in the dry-gas environment. In the clean room the longer equilibration was associated with a slightly higher $D_{110°C}$-value.

Table 5.2 - Effect of Equilibration Time on $D_{110°C}$-values of Three Spore Suspensions

Equilibration Time	$D_{110°C}$-value in Min. (No. of Trials)					
	Spores AAAB		Spores AANE		Spores AADB	
	Dry Gas	Clean Room	Dry Gas	Clean Room	Dry Gas	Clean Room
24 Hours	22.0 (1)	97.1 (2)	35.8 (1)	148.0 (2)	32.5 (4)	116.9 (1)
48 Hours	20.1 (1)	101.5 (2)	37.2 (1)	165.4 (2)	30.4 (4)	125.6 (1)

Experiment 3

Because the spore coded AAOE generally appeared to be at least as resistant as the other spore suspensions, additional work was done with that suspension. As the original suspension of those spores (AANE) had been stored in water at 4°C and the new suspension had been frozen during shipment, comparative $D_{110°C}$-values were obtained for the old, non-frozen suspension and the new, frozen suspension. The results of these experiments are summarized in Table 5.3. While results were not conclusive from the limited number of trials, there did not appear to be a major effect of freezing on the D-value determination.

Table 5.3 - Effect of Freezing on the $D_{110°C}$-value of a *Bacillus subtilis* var. *niger* suspension Stored in Water

	$D_{110°C}$-value in Min. (No. of Trials)	
Spore Suspension	Dry Gas	Clean Room
Frozen (AAOE)	41.7 (2)	133.7 (2)
Non-frozen (AADB)	30.9 (3)	141.3 (4)

Experiment 4

All experiments described previously utilized spore suspensions which had been pipetted onto stainless steel planchets to yield approximately 1×10^6 spores per deposit before heating. An experiment was carried out in which deposits of approximately 1×10^4 and 1×10^8 spores in equal volumes of water were also evaluated. The results of these tests are summarized in Table 5.4.

It appears that the $D_{110°C}$-values were slightly lower when the lowest concentration (10^4) was used. The very high concentration of spores was very difficult to work with. We hope to make additional comparisons between 10^4 and 10^6 spores per deposit in the future.

Table 5.4 - $D_{110°C}$-values of Bacillus subtilis var. niger Spores (Frozen Suspension B, Spores AAOE) as a Function of Initial Concentration of Spores per Deposit

Initial Concentration per Deposit	$D_{110°C}$-value in Min.	
	Dry Gas Environment	Clean Room Environment
10^4	21.8	136.0
10^6*	41.7	153.0
10^8	42.1**	143.7

* Based on all previous observations

** Based on only two data points

Experiment 5

The results of the series of tests at 110°C in the two conditioning and heating environments with nitrogen as well as air in the low humidity environment are listed in Table 5.5. The data for the several conditions are summarized in Table 5.6. The effect of the relative humidity in the gas surrounding the hot plate during both heating and conditioning is dramatic in that the average for the four "d" conditions (Table 5.5) is 161 minutes compared to an average of 43.3 minutes for the four "a" conditions. The results also suggest

Table 5.5 - Results of Dry Heat Destruction-Rate Studies of Bacillus subtilis var. niger Spores AAOE on Stainless Steel Surfaces as a Function of Conditioning and Heating Environment

Test Number		Conditioning		Heating		$N_o \times 10^{-6}$	$D_{110°C}$-value in Min.	Y-intercept $\times 10^{-6}$
		% RH at 22°C	Environment	% RH at 110°C	Environment			
	a	0.26-.98	Air	0.010-0.014	Air	1.72	43.1	.36
1	b	34-36	Clean Room	0.010-0.014	Air	2.03	40.4	.63
	c	0.26-.98	Air	0.65-0.68	Clean Room	1.72	140.7	1.60
	d	34-36	Clean Room	0.65-0.68	Clean Room	2.16	163.4	1.52
	a	0.29-0.72	Air	0.009-0.019	Air	1.92	42.3	.62
2	b	34-36	Clean Room	0.009-0.019	Air	2.23	42.5	.63
	c	0.29-0.72	Air	0.65-0.68	Clean Room	1.90	142.0	1.81
	d	34-36	Clean Room	0.65-0.68	Clean Room	2.52	166.6	1.30
	a	0.17-2.01	Nitrogen	0.005-0.008	Nitrogen	1.79	38.6	1.13
3	b	34-36	Clean Room	0.005-0.008	Nitrogen	1.92	43.3	.48
	c	0.17-2.01	Nitrogen	0.65-0.68	Clean Room	1.99	177.6	.56
	d	34-36	Clean Room	0.65-0.68	Clean Room	1.96	139.6	.71
	a	0.05	Nitrogen	0.0009	Nitrogen	1.72	49.1	.29
4	b	34-36	Clean Room	0.0009	Nitrogen	2.24	37.7	.52
	c	0.05	Nitrogen	0.65-0.68	Clean Room	1.85	147.7	1.45
	d	34-36	Clean Room	0.65-0.68	Clean Room	2.03	161.2	.76

that there is no difference in the D-value that can be associated with the type of dry atmosphere. Mean D-values for spores conditioned and heated in the dry gas environment were 42.7 minutes for the dry air and 43.9 minutes for the dry nitrogen. Mean D-values for spores conditioned in the clean room and heated in the dry-gas environment were 41.5 for dry air and 40.5 for dry nitrogen. The moisture-analysis readings indicate that the overall relative humidity range during the heating period was higher for the dry air than it was for dry nitrogen (0.009%-0.022% for air vs. 0.0009-0.008 for nitrogen). Thus it is possible that the D-values are lower for spores in air than for those in nitrogen for an equivalent relative humidity condition. Subsequent experiments will be carried out to explore that possibility.

Table 5.6 - A Summary of the Data in Table 5.5 Showing the $D_{110°C}$-values for the Replicate Tests as a Function of the Specific Environmental Condition

Heated		Conditioned	$D_{110°C}$-values in Min.
Dry Air	a	Dry Air	43.1, 42.3
	b	Clean Room	40.4, 42.5
Dry Nitrogen	a	Dry Nitrogen	38.6, 49.1
	b	Clean Room	43.3, 37.7
Clean Room	c	Dry Air	140.7, 142.0
	c	Dry Nitrogen	177.6, 147.7
	d	Clean Room	163.4, 166.6, 139.6, 161.2

It is apparent that in the two dry nitrogen experiments, the relative humidity effect did not operate in that manner. During the second experiment, the relative humidity was appreciably lower than during the first experiment (0.0009-0.0012 compared to 0.005-0.008), yet the D-value was considerably higher at the lower humidity (49.1 minutes vs. 38.6 minutes). It appears then that either the effect of minute quantities of water was more complex than anticipated or the technique for measuring humidity was sufficiently imprecise that it makes the data comparisons meaningless.

A comparison of the a vs. b data and the c vs. d data will give an indication of the effect of the conditioning treatment (initial level of water in the spores) on D-value. The average D-value for "a" Condition is 43.3 minutes and that for "b" condition is 41.0 minutes suggesting that the initial water content probably has no significant effect. Comparing c and d, the average for c is 152 minutes and that for d is 158 minutes; suggesting that again there is no significant effect of initial spore water content on D-value.

It is also interesting to note that in seven out of eight measurements N_o was lower following dry gas conditioning than it was after conditioning in the clean room. In addition, in three out of four of the experiments (1, 2, and 4) the y-intercept was lower following dry gas heating than it was following ambient environment heating.

CONCLUSIONS

The experiments to date, at 110°C have shown that heating in a dry-gas environment (<0.03% RH at 110°C) results in D-values considerably lower than those obtained by heating in an ambient environment (∿34-36% RH at 22°C or 0.65-0.86% at 110°C). A D-value ratio of approximately 1:4 was noted in almost every trial carried out.

A comparison of dry nitrogen and dry air did not reveal any difference between the two gases; although drier conditions were associated with nitrogen than with air. Thus it is possible that a difference might exist under equivalent moisture conditions.

There was an indication that heating in a dry gas may have reduced the y-intercept (indicating that a sharper than usual reduction took place during the first heating interval), compared to heating in the clean room.

FUTURE WORK

Priorities for additional experiments related to this task include the following:

1. Determination of D-values in the dry gas environment for Bacillus subtilis var.nniger (AAOE) at temperatures both

higher and lower than 110°C. Data will be obtained at 125°C and 90°C, again utilizing both dry air and dry nitrogen in comparison with ambient clean room environments.

2. Determination of D and Z-values in the range of 90-125°C for highly resistant spores isolated from Mariner '69.
3. Determination of the effect of varying relative humidity in the range of 25-45% on $D_{125°C}$-values as measured on a hot plate in a 22°C clean room.
4. Determination of the effect on $D_{125°C}$-values of varying the room temperature (16-26°C) and relative humidity (25-45%) while holding vapor pressure constant in the clean room.
5. Determination of $D_{125°C}$-values at a relative humidity intermediate between the 0.006% and 0.4% currently being achieved (as calculated at 125°C in the dry gas and clean room environments).
6. Determination of dry gas D-values for spores contained on surfaces other than stainless steel (e.g. glass, plastic, or painted surfaces).
7. Spore species other than Bacillus subtilis var. niger will also be studies to determine the consistency of the dry gas phenomena.

APPENDIX A: THE USE OF SILICA GEL PRE-LOADED WITH WATER TO PRODUCE A CONSTANT RELATIVE HUMIDITY IN A CLOSED CHAMBER

Introduction

Water content appears to be the most important variable in the dry heat destruction rate of microbial spores. One method of varying the water content of spores is to equilibrate the spores in chambers of different relative humidity.

Objective

Throughout the scientific world, saturated salt solutions are used to produce and maintain a fixed equilibrium relative humidity. We were concerned about minute quantities of salt being depositid with the spores on the metal strips. For this reason, we investigated other systems for producing a constant relative humidity. Silica gel pre-loaded with water appeared to have considerable merit for this application.

Our research group's decision to use silica gel to control the relative humidity of environmental cabinets grew out of conversations in March, 1968, with Dr. Elias J. Amdur, Design Supervisor, Apparatus Controls Division, Honeywell Corporation. The following important points resulted from those discussions:

1. Salt solutions often are not satisfactory for relative humidity control for the following reasons: 1) the solutions exhibit creep, i.e., a tendency to climb up the walls of their container, 2) in the usual application, when a container of salt solution is placed in the bottom of a jar, the equilibration time is very long, e.g. 24 hours for a one-gallon jar, and 3) the relative humidity sensing elements become contaminated when stored over salt solutions, even though they are protected from direct contact with the solution.
2. The Honeywell Corporation uses silica gel pre-loaded with water to produce controlled relative humidity levels. A curve of equilibrium relative humidity versus the quantity of water added can be developed for each lot of silica gel. The curve can be

reliably used to pre-load silica gel with water to a desired relative humidity.

3. Silica gel is less stable for moisture control over a long period of time than salt solutions since the relative humidity is a continuous function of the water added to the silica gel.
4. If a sterile humidity-control medium is desired, silica gel and water can be heat sterilized separately and mixed aseptically.
5. Air movement is needed in a humidity control chamber to prevent unduly long equilibration times. A fan is satisfactory but the motor should be kept outside the cabinet in order to avoid heat problems. Alternatively, a rheostat or other control may be used to lower power consumption or the cabinet may be designed to dissipate heat rapidly. To minimize air circulation requirements, the silica gel may be suspended in small packages at many locations in the controlled cabinet.
6. If a humidity cabinet is not temperature controlled, the quality of air conditioning in the surrounding room may cause problems. A 2°F variation in room temperature is likely in an air-conditioned room; in addition to causing fluctuations in the relative humidity, a 2° temperature change may cause condensation in a high relative humidity chamber.

Experimental Procedure - General Recommendations for Loading Silica Gel with Water

The loading procedure consists of adding the water to the silica gel in several portions in a sealed jar, mixing gently but thoroughly after each addition. An overnight (4-15 hours) stabilizing period should be allowed before the equilibrium relative humidity is checked and the gel put into use. Since there is a lot-to-lot variation in the water-loading characteristics of silica gel, the equilibrium relative humidity must be checked and a relative humidity versus water-loading curve developed for each lot of silica gel if accurate relative humidity conditions are to be produced. A typical loading curve from our laboratory is shown in Figure A-1.

The water-loaded silica gel should be stored and used at a constant temperature.

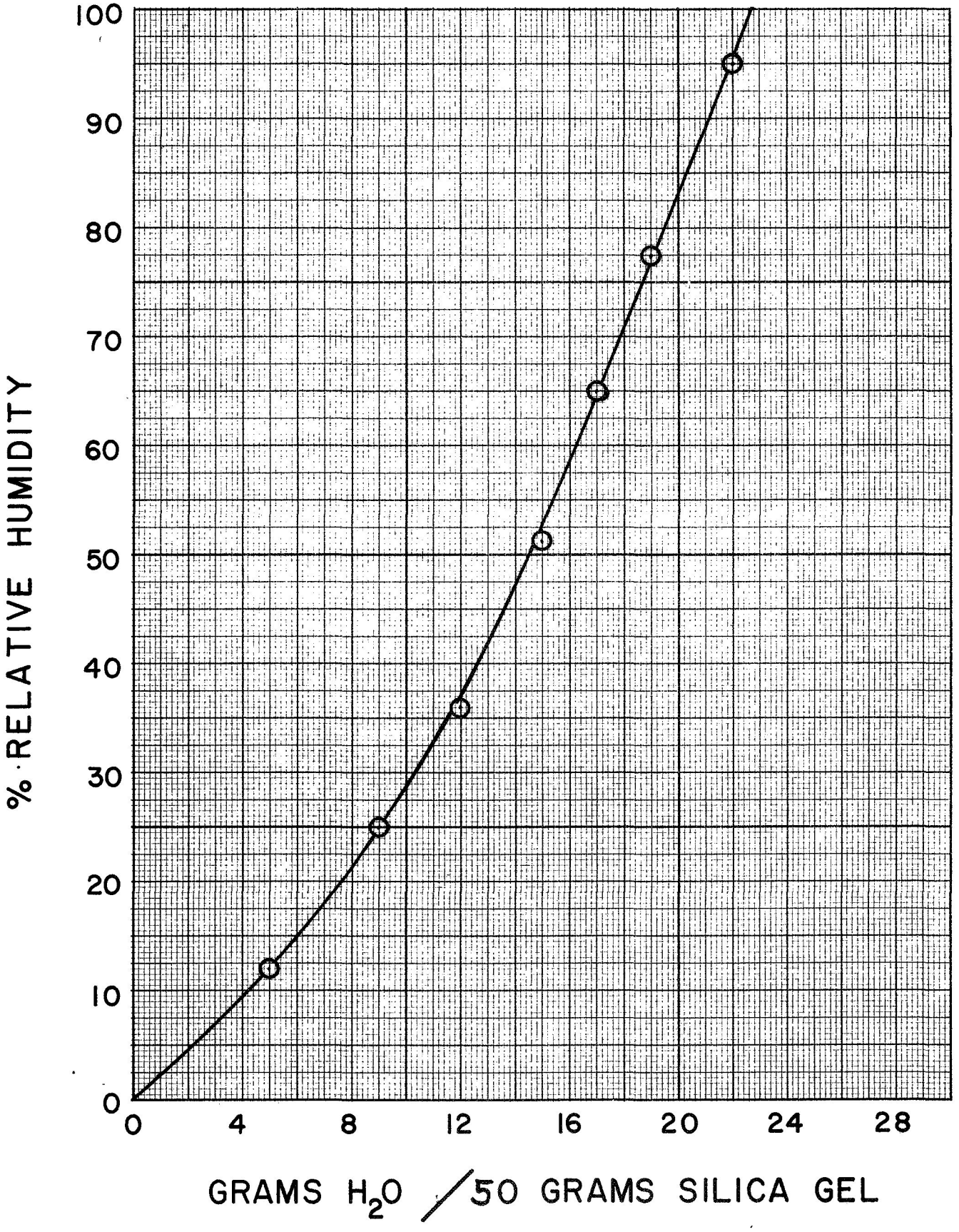

Figure A-1 - Relative humidity as a function of the quantity of water added to one lot of silica gel. Temperature 45°C

In our laboratories, silica gel loaded with water has been used to produce a controlled relative humidity environment in both small plastic (200 ml) and glass (500 ml) containers and in a small (0.2 m^3) plastic glove box.

Reuse of the gel is not recommended since the relative humidity reproducibility decreases after the first use.

Procedures for Small Plastic and Glass Containers

Refrigeration-grade silica gel, mesh size 8-20 is used. The gel is dried in a forced air oven at 100°C, for 24 hours, weighed into screw cap bottles, reheated to 100°C, for 24 hours, tightly capped and stored until used.

Twenty-four hours before setting up the relative humidity chamber the correct amount of distilled water is added to the silica gel. Thorough mixing is accomplished by gentle agitation of the bottle. Hard shaking is not recommended since it appears to break up the crystals. The water and gel are then equilibrated for 24 hours before being added to the humidity chamber.

The boxes are fitted with a bulkhead connector and relative humidity-temperature sensor so that humidity can be measured without opening the inner box. After the addition of 50 grams of silica gel plus the appropriate amount of water to the box, the cover is taped in place with masking tape; the box is then heat sealed in double polyethylene bags and placed in a controlled-temperature chamber.

The plastic bag is cut open at intervals to measure the relative humidity (a Honeywell Indicator, #W6111A was used for the relative humidity measurement.) The boxes are immediately resealed in plastic bags. The taped box itself is not opened.

The reproducibility of a specific relative humidity in replicate boxes varies with the humidity. The actual relative humidity recorded over a two-month period on boxes intended to be at <2%, 50% and >90% is shown in Table I. On replicate boxes dry silica gel always produced a relative humidity of less than 2% while at higher humidities, reproducibility was ±5%.

Table I - Relative Humidity Levels in Individual 5 1/4", 3 1/2" and 2 1/2" Plastic Boxes at 45°C as a Function of Time During an Experiment

Time	Box #1	Box #2	Box #3
Desired RH	<2%	50%	90%
After 2 days	<2%	55%	97%
After 30 days	<2%	56%	100%
After 60 days	3%	57%	96%

Procedure for Glove Box

Silica gel pre-loaded with water has been used to produce and maintain a controlled humidity condition in plastic glove boxes of about 0.2 cubic meter volume. Stainless steel pans were fastened together and placed on the floor of the glove box so that the entire floor area of the glove box was covered. 900 grams of silica gel pre-loaded with water was spread in a thin layer over the pans. A perforated expanded metal floor was placed over the pans to provide a surface on which the samples could be placed for equilibration.

To insure that the temperature and humidity remained uniform throughout the glove box, a four-inch diameter propeller fan circulating 200 cfm was mounted in each box. Although the fan was operated at full-line voltage the heat output from the fan produced a temperature rise of less than 2°C. In the normal use of the glove box, the controlled relative humidity level is reestablished in about thirty minutes after the glove box is opened for addition or removal of samples.

Results

The system has been used over a five-month period during which period the relative humidity in the room in which the glove boxes were located was very low. As might be expected, the low humidity box maintained a less than 2% relative humidity. The high humidity glove box, however, required weekly additions of water to maintain a constant relative humidity level. The amount of water required varied from week to week and was determined by trial each time. A relative humidity above 92% could not

be reliably attained. When the humidity was above 92%, water condensed on the wall of the plastic glove box because the temperature in the room surrounding the box was a few degrees lower than the temperature inside the glove box.

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APPENDIX B: CODING SPORE CROPS AT THE UNIVERSITY OF MINNESOTA

Introduction

Organization is the foundation of science and understanding. One researcher working with two or three spore crops has a few problems in identifying his spores and keeping track of the results from the different spore crops. When there are people in several laboratories working with more than ten different spore crops, it is necessary to codify these spores so that the identification of all crops is similar. Provision is made in the system for a large number of variations in spore crops with a continuity of identity. Some of the variables that have to be included are the basic genus and species of the culture, the place of isolation or the source, the methods of culturing the organism and perhaps the final suspending medium. We have attempted to put together a spore code that will allow us to fit all of our spore crops into one identification system. The continuity of this system is such that when we look at data from different spore crops the code letters will allow us to have a feel for the spores without having to go back and look up the details on each spore crop.

Explanation of the Spore Crop

The code consists of four letters. The first letter designates the genus, species and subspecies of the organism if they are known. If the spore is an unknown, any identifying number and the place of isolation are given.

The second letter designates the place from which this laboratory originally received the spores.

The third letter identifies the specific spore crop. Information includes date of the crop, culture place and method, cleaning procedures, suspending fluid and any other pertinent information.

The fourth letter designates the storage conditions in this laboratory.

Coded Spore Crops Used in this Progress Report

AAAA

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix, Arizona

A Date cultured: August, 1968

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: 95% ethanol

A 95% ethanol, -20°C

AAAB

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix, Arizona

A Date cultured: August, 1968

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: 95% ethanol

B 95% ethanol, -20°C, resuspended in deionized distilled water at +4°C

AAAC

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix

A Date cultured: August, 1968

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: 95% ethanol

C 95% ethanol, -20°C, resuspended in deionized distilled water at -20°C

AABF

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix

B Date cultured: August, 1968

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: Deionized distilled water

F Deionized distilled water at +4°C

AADB

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix

D Date cultured: June, 1969

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: 95% ethanol

B 95% ethanol, -20°C, resuspended in deionized distilled water at +4°C

AAND

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix

N Date cultured: March, 1969

Place cultured: USPHS, Phoenix, Arizona

Culture procedure: No. 2

Suspending fluid: Buffered distilled water

Date received: March, 1969

Condition when received: Packed in ice

D Buffered distilled water at +4°C

AANE

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix

N Date cultured: March, 1969

Place cultured: USPHS, Phoenix, Arizona

Culture procedure: No. 2

Suspending liquid: Buffered distilled water

Date received: March, 1969

Condition when received: Packed in ice

E Buffered distilled water at +4°C, resuspended in deionized distilled water at +4°C

AAOE

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix

O Date cultured: March, 1969

Place cultured: USPHS, Phoenix, Arizona

Culture procedure: No. 2

Suspending fluid: Buffered distilled water

Date received: September, 1969

Condition when received: Packed in dry ice, frozen

E Buffered distilled water at +4°C, resuspended in deionized distilled water at +4°C

VDLA

V Unknown, Isolate #4-47A, Mariner '69

D Jet Propulsion Lab

L Date cultured: October, 1969

Place cultured: JPL, Pasadena, California

Culture procedure: No. 2

Suspending fluid: 95% ethanol

Date received: December, 1969

Condition when received: Packed in dry ice

A 95% ethanol, -20°C

VDLB

V Unknown, Isolate #4-47A, Mariner '69

D Jet Propulsion Lab

L Place cultured: JPL, Pasadena, California

Culture procedure: No. 2

Suspending fluid: 95% ethanol

Date received: December, 1969

Condition when received: Packed in dry ice

B 95% ethanol, - 20°C, resuspended in deionized, distilled water at +4°C

Procedure No. 1 - Spore Production Using Supplemented TAM Sporulation Medium

1. Use Difco TAM Sporulation Agar #0892. The medium is prepared according to the directions on the bottle. Prior to pouring the medium into the plates it is supplemented with two salt solutions:
   a. Calcium chloride: Make a 10% $CaCl_2$ solution, filter sterilize and add 0.8 ml per liter after medium has cooled.
   b. Magnesium sulfate: Make a 10% $MgSO_4$ solution, filter sterilize and add 0.2 ml per liter after cooling.

2. Heat shock an aqueous suspension of spores at 80°C for fifteen minutes. Using the heat shocked suspension inoculate the surface of a supplemented TAM plate with a cotton swab. Incubate at 41°C for 24 hours or until sporulation occurs.
3. Prepare a heavy suspension of spores from the above TAM plate in sterile deionized distilled water and heat shock at 80°C for fifteen minutes. Inoculate a fresh plate of TAM with a cotton swab and incubate at 41°C for three hours.
4. Swab the above plate and inoculate another plate of TAM that has been equilibrated at 41°C. Incubate for three hours and repeat this procedure.
5. You should now have a culture that is in the log phase of growth. Use this plate to inoculate ten plates of TAM that are at 41°C. Incubate at 41°C for at least 24 hours. Check each plate microscopically for sporulation. Any plate that does not have at least 90% sporulation after 48 hours should be discarded.
6. Collect the growth from the plates using a minimal amount of cold sterile deionized distilled water. Using a Branson Sonogen A Series ultrasonic bath, insonate in cold 0.3% Tween 80 for thirty minutes at 25 Khz/sec. to break up vegetative cells. The temperature of the bath is monitored and maintained at 5-10°C by the addition of ice.
7. Centrifuge the suspension at 2400 rpm, 4°C using a refrigerated centrifuge. The spores will separate from the cellular debris. Wash and recentrifuge six times. If the final suspension is to be stored in 95% ethanol, rinse twice in ethanol.
8. The final spore suspension is checked microscopically by making a smear and staining by the Schaeffer and Fulton Modification of the Wirtz Method of Staining Bacterial Spores.

Procedure No. 2 - Spore Production Using Synthetic Sporulation Medium-10
(This medium was described by Lazzarini and Santangelo, J. Bact.94:125-13C

1. This procedure using Synthetic Sporulation Medium-10 is a liquid culture system. The following materials with the exception of the metal salts are dissolved in 900 ml of distilled,deionized water

and sterilized for fifteen minutes at 121°C. The metal salt solution is made up in ten-fold concentration. 100 ml are added aseptically to the base medium after it has cooled

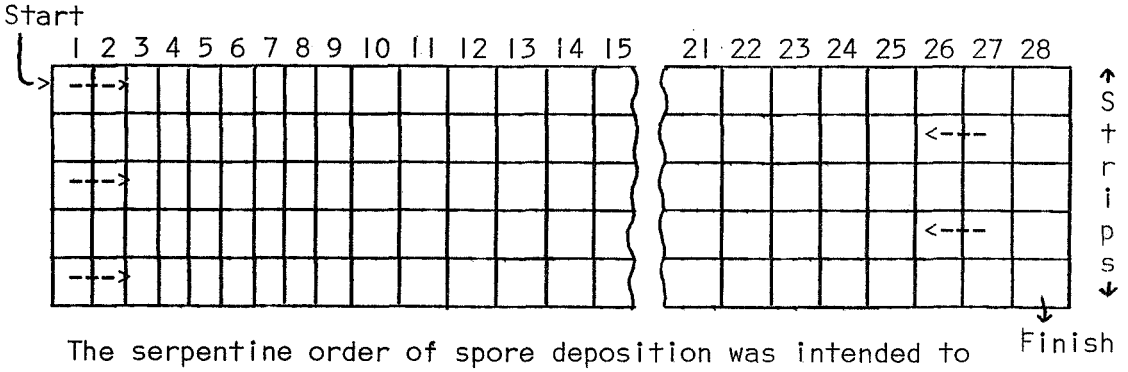
	Per liter
$K_2HPO_4 \cdot 3H_2O$ (K_2HPO_4)	7.3 gm
KH_2PO_4	2.4 gm
Sodium Citrate	1.0 gm
Tris (hydroxymethyl) Aminomethane (free base)	3.6 gm
Glutamic acid	4.4 gm
Glucose	1.5 gm
Tryptophan	25.0 mg
Methionine	25.0 mg
$CaCl_2$ ($CaCl_2 \cdot 2H_2O$)	111.0 mg
$MgSO_4 \cdot 7H_2O$	246.5 mg
$ZnCl_2$	6.81 mg
$MnCl_2 \cdot 4H_2O$	9.89 mg
$FeCl_3$	2.70 mg

2. Three hundred ml portions of the complete medium in Bellco one liter baffled flasks are inoculated with 5 ml of a 24-hour SSM-10 broth culture. Flasks are then incubated at 136 rpm and 32°C for five days (ca. 90% spores) in a Psychro-Therm Incubator Shaker (New Brunswick Scientific Co.).
3. After incubation, growth in 800 ml of medium is harvested with a refrigerated continuous flow centrifuge at 800 x g. Pellets are polled, placed in an ice bath and exposed to ultrasonic energy using a probe operating at 60% maximum intensity for 90 seconds, to disrupt clumps and cell debris.
4. The spore suspension is then recentrifuged and washed four times. If the spores are to be stored in ethanol, rinse them twice with ethanol.
5. The final spore suspension is checked microscopically by making a smear and staining by the Schaeffer and Fulton Modification of the Wirtz Method of Staining Bacterial Spores.

APPENDIX C: METHOD OF RANDOMIZATION OF TREATMENT CONDITIONS

(This example is for Experiment 5. In other experiments the method was the same but treatment combinations differed.)

1. Each trial utilized 28 permanently-numbered rectangular copper boats each containing a row of five 1/2"-square,sterile, stainless steel* strips (planchets) which lay flat in a shallow groove running the length of each boat.
2. The boats were arranged in numerical order, parallel to each other on a clean stainless steel sheet.
3. By means of a twenty µl Eppendorf Microliter Pipette** 0.02 ml of aqueous spore suspension ($\sim 1 \times 10^6$ spores) was deposited onto each strip in a serpentine manner starting at the upper left-hand corner and continuing to the upper right, following the diagram as indicated:



The serpentine order of spore deposition was intended to reduce between-boat variation at the possible expense of increased within-boat variation.

4. There were four treatment combination groups: (RH measured at 22-24°C)
   a. <1% RH conditioning and <1% RH heating
   b. 35% RH conditioning and <1% RH heating
   c. <1% RH conditioning and 35% RH heating
   d. 35% RH conditioning and 35% RH heating

*Type 302 fullhard spring-temper stainless steel shim stock, 0.015-inch thickness

**See Procedure for the Deposition of a Spore Suspension by Use of the Eppendorf Microliter Pipette (Appendix D)

5. For each treatment-combination group, (a thru d) seven copper boats were randomly assigned. Following is a sample random-allocation of boats to treatment.

Treatment	Copper Boat Number			
	a	b	c	d
N_o	11	1	28	3
1st heating time	2	16	19	13
2nd heating time	18	25	4	22
3rd heating time	24	17	26	7
4th heating time	9	15	14	10
5th heating time	27	5	20	23
Spare	12	6	21	8

Four of the five planchets were processed from each copper boat. The center one was not analyzed unless another was lost, up to the point where plates were placed safely on the incubator shelf. Experience to date indicates that this safety margin was adequate.

APPENDIX D: PROCEDURE FOR THE DEPOSITION OF A SPORE SUSPENSION BY USE OF THE EPPENDORF MICROLITER PIPETTE*

1. A sterile, dry tip was placed over the conical lower part of the pipette (yellow for 5 to 100 µl, red for 101 to 1000 µl).
2. The spore suspension was mixed on the Vortex Mixer, maximum power for five to six seconds. The pipette was rinsed once with spore suspension before making the first deposit. It was mixed again after every nine deposits, the cover was replaced before mixing.
3. The stem of the pipette was grasped so that the thumb could operate the colored button on top.
4. The pushbutton was depressed to first stop, then the tip was immersed slightly into the spore suspension.
5. The pushbutton was allowed to return to starting position;solution entered the tip.
6. The tip was withdrawn from the spore suspension and moved slowly along the well of the tube; the edge was touched slightly to remove excess.
7. The tip was placed close to the surface of the stainless steel strip.
8. The pushbutton was depressed to first stop, and after one second it continued all the way down to the second stop.
9. With the pushbutton remaining fully depressed the pipette was slowly withdrawn. The spore deposit was not spread so as to obtain maximum challenge effect.
10. The pushbutton was allowed to return to starting position.

*The filled pipettes were always held upright. A special rack was used for temporary upright storage.

APPENDIX E: GUIDELINES FOR THE ESTIMATION OF THE NUMBER OF SURVIVING SPORES FOLLOWING DRY HEAT TREATMENT

1. Count colonies on all plates and record plates with >600 colonies as TNTC.
2. Do not use any plated aliquot which has a TNTC count or a zero count in any calculation.
3. When making calculations, if possible, use results from that dilution yielding a mean count (mean of 8 colony counts; 2 each from 4 strips) in the range of 30-300 (refer to Standard Methods of Dairy Products, 12th Edition, 1967.) If two such dilutions appear, use the one which yields the highest extrapolated mean count.
4. If no such dilution appears, then use a dilution with a mean of 20 or greater, or less than 500. If there are two such dilutions, again use the highest extrapolated mean.
5. Under no circumstances are data from two or more different aliquots used. If no dilution yields applicable counts, that data time-point is considered lost.

APPENDIX F: GUIDELINES FOR QUALITY CONTROL OF CULTURE MEDIUM (TRYPTICASE SOY AGAR, BALTIMORE BIOLOGICAL LABORATORIES, PRODUCT NUMBER 11043)

1. Record the lot number of the dehydrated medium.
2. When sterilizing a batch of medium, which has been prepared according to the specifications of the manufacturer*, place a maximum-registering thermometer in the chamber of the autoclave during the sterilization cycle. (Capillary contraction allows the mercury to rise when heated but prevents it from receding until it is shaken down.) Remove it from the autoclave at the completion of the sterilization cycle, and allow it to cool to room temperature before reading. (Errors may result if reading is taken before thermometer has cooled.) A vacuum exists above the mercury column and readings must be made with the thermometer in upright position. Record the reading along with an assigned batch number on a separate sheet of paper. Indicate the specific sterilization cycle on the recording chart which indicates temperature and time and save the chart. Periodically check the accuracy of all thermometers used against a standard. Check the recorded time of the sterilization cycle by actual observation of built-in thermometers. If discrepancies occur, adjust the autoclave to correlate with the stated time and temperature of the chart and the stated pre-set temperature of the chamber during the sterilization cycle.
3. Store the prepared medium in the refrigerator at 4°C, in tightly-closed, screw-cap tubes, for a period not exceeding one month.
4. Melt the medium on the same day it is to be used. (Medium melted at the beginning of the day is used at the beginning of plating for that day.)
5. When melting tubes of medium, use a fixed time; in the large autoclave, five minutes; in the small autoclave, three minutes. Upon completion of the melting cycle, transfer the tubes immediately to the media conditioner (50-53°C) or water bath (50-53°C). Any tubes with a decrease in volume should be discarded.
6. Any tubes of medium containing a precipitate should be discarded, as their use may result in reduced capacity for growth promotion.

Precipitation is usually a result of overheating.

7. Discard unused tubes of melted medium at hhe end of the working day and do not remelt prepared medium more than once.
8. Prepare all media used in a single experiment from the same "lot" of dehydrated media.

*Method of Preparation: Suspend 40 grams of the powder in a liter of distilled water and mix thoroughly. Heat with frequent agitation, boil for one minute and dispense and sterilize at 118°C to 121°C (not over fifteen pounds of steam pressure for fifteen minutes. Do not overheat.)

PUBLICATIONS AND PRESENTATIONS

Publications in the Open Literature

Greene, V. W. and D. Vesley. 1969. Microbial Contamination Control in Hospitals, Part 4 - Role of Housekeeping. Hospitals. 43(23): 69-80.

Presented at Meetings

Vesley, Donald. "Cooperative Microbial Evaluation of Hospital-Patient-Room Floor Cleaning Procedures," Report of APHA Laboratory Section Committee on Microbial Contamination of Surfaces at the annual meeting of the American Public Health Association, November 11, 1969.